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Escola Superior de Tecnologia da Saúde do Porto

Instituto Politécnico do Porto

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Macrophages as biomarkers in BCG treatment response in bladder cancer

Mestrado em

Tecnologia Bioquímica em Saúde

Setembro de 2013

ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DO PORTO

INSTITUTO POLITÉCNICO DO PORTO

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Dissertação submetida à Escola Superior de Tecnologia da Saúde do Porto para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Tecnologia Bioquímica em Saúde, realizada sob a orientação científica do Professor Doutor José Alexandre Ferreira, Investigador de Pós-Doutoramento do Centro de Investigação do Instituto de Oncologia do Porto (IPO- Porto) e do Departamento de Química da Universidade de Aveiro e co-orientação da Professora Doutora Cristina Prudêncio da Escola Superior de Tecnologia da Saúde do Porto e Mestre Luís Lima Investigador de Doutoramento do Centro de Investigação do Instituto de Oncologia do Porto (IPO- Porto).

Setembro, 2013

Agradecimentos

Aos meus orientadores Luís Lima e Alexandre Ferreira pela confiança que depositaram em mim aceitando me para orientação deste projeto. Pela disponibilidade e apoio, pelo incentivo e ensinamentos, por terem enriquecido os meus conhecimentos científicos e me terem ajudado a crescer enquanto pessoa e pela forma amável como me acolheram no Grupo de Terapêutica e Patologia Experimental.

A todos os restantes membros do Grupo de Terapêutica e Patologia Experimental pela forma carinhosa com que me acolheram e por todo o acompanhamento. À Elisabete especialmente pelos conselhos e disponibilidade para esclarecer todas as minhas dúvidas de trabalho e existenciais.

Às minhas meninas do mestrado todo acompanhamento e ajuda.

Ao meu querido namorado, Daniel Neto, pelo apoio que me deu em todos os momentos. Pela paciência que teve e por ter estado sempre presente quando precisei.

Aos meus pais e irmão pelo carinho, pelo apoio incondicional demonstrado ao longo de todo o meu percurso escolar. Sem vocês não era nada.

A todos o meu sincero,

Obrigada!

Resumo

O cancro da bexiga é um cancro urológico comum e a maioria tem origem no urotélio. Pacientes com risco intermediário e alto de recidiva / progressão do cancro da bexiga são tratados com instilação intravesical com Bacillus Calmette-Guérin (BCG), no entanto, aproximadamente 30% dos pacientes não respondem ao tratamento. No momento, não há biomarcadores para prever o resultado do tratamento e uma identificação precoce dos pacientes por terapias alternativas.

O tratamento inicia uma cascata de citocinas responsáveis pelo recrutamento de macrófagos para o local do tumor que têm mostrado influenciar o resultado do tratamento. A terapia eficaz ao BCG necessita de ativação precisa da via imune Th1 associada com polarizados em macrófagos M1. No entanto, *tumor-associated macrophages* (TAMs) assumem um fenótipo M2 imunoregulador, tanto imunossupressivo ou angiogénico, que interferem em diferentes maneiras com a resposta imunológica antitumoral induzida por BCG. O macrófago M2 é influenciado por diferentes microambientes no estroma e do tumor. Em particular, o grau de hipoxia é responsável pelo recrutamento e diferenciação dos macrófagos para o fenótipo angiogénico M2 e pode estar relacionado com a resposta ao tratamento. No entanto, nem os fenótipos de macrófagos, nem a influência da localização e hipoxia foram abordados em estudos anteriores.

Assim, este trabalho é dedicado ao estudo da influência de TAMs, em particular do fenótipo M2 tendo em conta a sua localização (estroma ou tumor) e o grau de hipoxia no tumor (baixa ou alta) em resultado do tratamento com BCG.

O estudo incluiu 99 pacientes com cancro da bexiga tratados com BCG. Os tumores ressecados antes do tratamento foram avaliados usando imunohistoquímica para os antigénios CD68 e CD163, que identificam um marcador de linhagem de macrófagos e um receptor específico da superfície celular M2 polarizado, respectivamente. A hipoxia no tumor foi avaliada com base na expressão de HIF- 1α. Como principal conclusão, observou-se que uma elevada predominância de contagens $CD163^{+}$ de macrófagos no estroma de tumores sob baixo nível de hipoxia foi associado com insuficiência da imunoterapia BCG, possivelmente devido ao seu fenótipo imunossupressor. Este estudo reforça ainda mais a importância do microambiente tumoral na modulação das respostas ao BCG.

Palavras-chave: Cancro da bexiga, Imunoterapia Bacillus Calmette-Guérin, Tumor-Associated Macrophages (TAMs), Macrófagos com fenótipo M2

Abstract

Bladder cancer is a common urologic cancer and the majority has origin in the urothelium. Patients with intermediate and high risk of recurrence/progression bladder cancer are treated with intravesical instillation with Bacillus Calmette-Guérin, however, approximately 30% of patients do not respond to treatment. At the moment, there are no accepted biomarkers do predict treatment outcome and an early identification of patients better served by alternative therapeutics.

The treatment initiates a cascade of cytokines responsible by recruiting macrophages to the tumor site that have been shown to influence treatment outcome. Effective BCG therapy needs precise activation of the Th1 immune pathway associated with M1 polarized macrophages. However, tumor-associated macrophages (TAMs) often assume an immunoregulatory M2 phenotype, either immunosuppressive or angiogenic, that interfere in different ways with the BCG induced antitumor immune response. The M2 macrophage is influenced by different microenvironments in the stroma and the tumor. In particular, the degree of hypoxia in the tumors is responsible by the recruitment and differentiation of macrophages into the M2 angiogenic phenotype, suggested to be associated with the response to treatment. Nevertheless, neither the macrophage phenotypes present nor the influence of localization and hypoxia have been addressed in previous studies.

Therefore, this work devoted to study the influence of TAMs, in particular of the M2 phenotype taking into account their localization (stroma or tumor) and the degree of hypoxia in the tumor (low or high) in BCG treatment outcome.

The study included 99 bladder cancer patients treated with BCG. Tumors resected prior to treatment were evaluated using immunohistochemistry for CD68 and CD163 antigens, which identify a lineage macrophage marker and a M2-polarized specific cell surface receptor, respectively. Tumor hypoxia was evaluated based on HIF-1α expression. As a main finding it was observed that a high predominance of $CD163^{+}$ macrophage counts in the stroma of tumors under low hypoxia was associated with BCG immunotherapy failure, possibly due to its immunosuppressive phenotype. This study further reinforces the importance the tumor microenvironment in the modulation of BCG responses.

Keywords: Bladder Cancer, BCG Imunotherapy, Tumor-Associated Macrophages (TAMs), M2 macrophages phenotype

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Abbreviations

AUA – American Urological Association

BCG – Bacillus Calméte-Guérin

BC – Bladder Cancer

CIS – Carcinona *in Situ*

CCL – Citocina CCL

EGF – Epidermal Growth Factor

EAU – European Association of Urology

HIF – Hypoxia Inducible Factor

IL – Interleucina

M-CSF – Macrophage Colony-Stimulating Factor

MMPs – Matrix Metalloproteases

MMC – Mitomycin C

NK – Natural Killer Cells

NMIBC – Non-Muscle Invasive Bladder Cancer

PDGF – Platelet Derived Growth Factor

STAT 3 – Signal Transducer and Activator of Transcription 3

SWOG – Southwest Oncology Group

TGF-β – Transforming Growth Factor Beta

TURBT – Transurethral Ressection of Bladder Tumors

TAMs – Tumor-Associated Macrophages

TNF-α – Tumor Necrosis Factor Alpha

VEGF – Vascular Endothelial Growth Factor

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CHAPTER I

Literature Review

1. Bladder Cancer

Bladder cancer (BC) is a common urologic cancer and the majority has origin in the urothelium. BC has the highest recurrence ratio of any malignancy.(1) The mean age of diagnosis is around 65 years. It is 3 times more common in men than in women, but has a worse prognosis in women.(2)

BC is the fifth most common cancer in the United States and one of the ten deadliest. It ranks fifth among all cancers in total costs, almost $3 billion annually, and has a wide effect on quality of life for survivors and their families.(3)

Risk factors that are normally associated with the development of bladder cancer include smoking, occupational exposure to chemicals, chronic infection and chemotherapeutic agents such as cyclophosphamide.(2) Smoking increases about 4 times the probability of developing bladder cancer, and after quitting smoking probability takes about 20 years to decrease or return to normal.(2) It is thought that exposure to carcinogenic chemicals are the major responsible for BC in men in Europe.(2)

The early, non-invasive diagnosis of BC is very difficult to obtain due to its specificity and the absence of specific markers. Hematuria is the first symptom and occurs in 85% of patients. However, only 7% of patients with hematuria have malignant tumors of the genitourinary system. Currently, the *American Urological Association* (AUA) and the *European Association of Urology* (EAU) recommends a combined diagnostic of a cystoscopy and cytology of cells obtained from bladder washing to achieve an accurate diagnosis.(3)

Cystoscopy is considered to be effective for detecting papillary tumors,(2) while the urine cytology is highly specific for the presence of high grade tumors or carcinoma *in situ* (CIS), however, the sensitivity is lower for low grade tumors. Positive urinary cytology can indicate an urothelial tumor anywhere in the urinary tract, from the calyx to the ureters, bladder and proximal urethra.(1, 4)

Approximately 75% to 85% of all BC are Non-Muscle invasive Bladder Cancer (NMIBC), CIS and superficial papillary confined to the mucosa or submucosa (Ta/T1). The bladder tumors which invades submucosa, mucosa, muscularis or adjacent organs (stage T2 or higher) are classified as Muscle Invasive Bladder Cancer (MIBC).(3,5)

CIS is a poorly differentiated carcinoma confined to epithelium with an intact basement membrane.(2) It can exist alone, although is usually present in association with other bladder tumors (90%).(1) Despite, CIS non-invasive nature it has a poorer prognosis than other superficial tumors, about 60% of patients progress to muscle-invasive disease over 5 years if left untreated.(2) Papillary tumors, confined to the mucosa, are classified as stage Ta according to the TNM system. Tumors that have invaded the *lamina propria* are classified as stage T1. Ta and T1 are grouped under the heading of non-muscle-invasive bladder cancer for therapeutic purposes.(4)

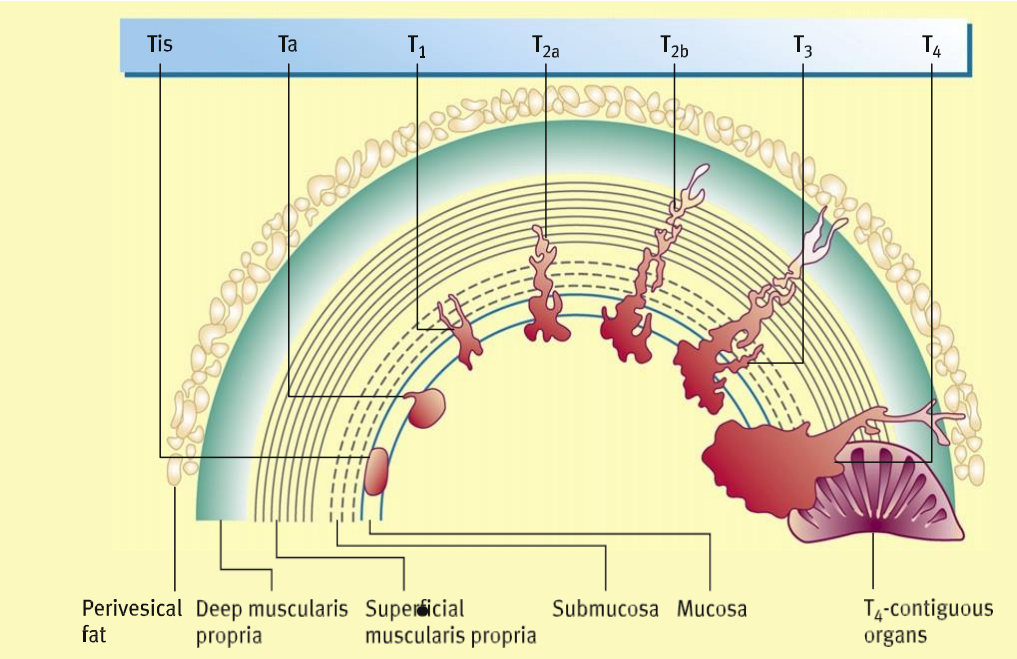

Figure 1 – TNM staging of transitional cell carcinoma bladder cancer based on invasion (T). (Adapted from (2))

The first-line treatment of a BC is transurethral resection of the bladder tumor (TURBT), comprehending a representation of the *muscularis propria* to ensure a total and a proper resection, and more precise staging.(1, 3, 5) After TURBT, patients are reviewed at a multidisciplinary team meeting and assigned a risk category.(1)

The NMIBC risk classification divides patients into low risk, intermediate risk and high-risk categories for recurrence and progression.(1) Low risk is defined as solitary, primary low grade Ta tumors; intermediate risk as multiple or recurrent low grade tumors; and high risk as any T1and/or CIS.(6) The scoring system is based on the six most significant clinical and pathological factors: number of tumors, tumor size, prior recurrence rate, T-category, presence of concurrent CIS and tumor grade.(4)

Patients with low risk disease are kept under cystoscopy surveillance. For patients with recurrent low grade disease or intermediate/high risk disease adjuvant intravesical therapy is recommended.(1) The gold standard treatment for intermediate/high risk patients is intravesical instillations with Bacillus Calmette-Guérin (BCG).(1) However, 30–50% of patients fail to respond, and 15% show progression to muscle-invasive disease. In these cases, radical cystectomy is the treatment to follow.(7)

2. Bacillus Calmette-Guérin (BCG)

Bacillus Calmette-Guérin (BCG) is an attenuated strain of *Mycobacterium bovis* that is used in bladder cancer as treatment, and instilled intravesically.(1) The key element of the BCG antitumor activity, lies in its ability to promote a strong local immune cell response(8), still the exact mechanism remain unknown.(5)

The immune complex cascade begins with the initial adhesion and internalization of mycobacteria to the urothelium and proceeds through the secretion of cytokines from urothelial and immune cells, a process that recruits a wide variety of inflammatory cells (neutrophils, monocytes, T cells) and cytokines to the tumor site and surroundings.(8)

Intravesical BCG immunotherapy is a standard treatment for CIS disease and high-grade or highly recurrent superficial tumors.(1) Mitomycin C (MMC) chemotherapy is another treatment that is recommended in patients with low and intermediate risk, where both agents are equally effective in reduction of recurrence but MMC has lower toxicity.(9)

In a meta-analysis of 7 studies comparing TURBT alone versus TURBT plus intravesical BCG, has shown that BCG significantly reduces recurrence in 12% and the chances of progression are reduced in 24%.(4) Another meta-analysis with 24 studies mentions a reduction in 27% the chances of progression when the patients received TURBT plus intravesical BCG.(1)

However, BCG immunotherapy for bladder cancer has its limitations. Even though of severe adverse effects are rare (<5%), approximately 90% of patients experience light cystitis and about one fifth are unable to tolerate BCG therapy. Various approaches have been tried in efforts to reduce side effects, for example the use of different dosages.(9, 10)

Induction BCG therapeutic scheme generally begin at least two weeks after TURBT to allow the healing of the urothelium and to reduce the risk of systemic side effects.(8) BCG therapy consists of a single course of six weekly intravesical instillations. The extension of treatment (maintenance immunotherapy) is used to increase efficacy.(11) The maintenance regimen consists in of three weekly instillations at 3 months, 6 months, and then every 6 months up to 3 years. (6, 8, 12) The adoption of maintenance schedule has been a crucial factor for improving BCG efficacy.(6,8)

A prospective study by the Southwest Oncology Group (SWOG) was performed to evaluate the efficacy of additional maintenance schedule following the initial induction cycle. This study demonstrated a significantly reduction of tumor recurrences in favor of the additional maintenance treatment. Furthermore, recent meta-analyses demonstrated that bladder tumor progression can only be prevented when an additional BCG maintenance (mBCG) therapy is applied.(12) In 20 trials analyzed, in some of which the mBCG scheme was used, it was observed a 37% reduction in the odds of progression.(4) Supposing that maintenance therapy is necessary for optimal efficacy, the problem of BCG toxicity becomes more significant. As a result of the more pronounced side effects of BCG, there is still hesitancy about the use of mBCG.(4) More than 70% of patients develop local symptoms and up to 37% develop systemic symptoms with maintenance therapy with BCG.(1)

BCG is currently considered the most effective intravesical agent for preventing recurrence in NMIBC, however 30-50% of patients do not respond, and 15% have the progression to invasive muscle disease.(7, 8, 13) When BCG immunotherapy fails, immediate radical cystectomy is recommended to reduce the risk of tumor progression.(6, 9)

At this moment, no valid prognostic markers have been found to predict clinical responses to BCG or to identify patients who may progress after BCG treatment. While studies on the mechanism of BCG provide several promising candidates such as interleucine-2 (IL-2), IL-8, IL-12, inflammatory chemokines (macrophages), none of these parameters are considered sufficiently consistent for daily practice. However, it is believed that immunological prognostic markers may yield promising clinical value in the context of predicting BCG immunotherapy outcome.(12)

3. Tumor-Associated Macrophages (TAM)

TAMs are originated from circulating blood monocytes recruited into the tumor. Their recruitment and survival *in situ* are directed by cytokines that interact with tyrosine kinase receptors.(14) The strategic location of TAMs, suggests that these cells are important regulators of anti-tumor immunity. Phenotypical characterization of TAMs is crucial to the understanding of tumor-derived signals guiding polarization of innate and adaptive immunity in cancer and to the identification of molecular mechanisms.(15)

The capability to express diverse functional programs in response to different micro-environmental signals is a biological feature of macrophages, which is typically demonstrated in pathological conditions such as infections and cancer.(15) TAMs can regulate the growth of various cancers, positively or negatively, because of their implication in defense against mycobacteria.(13, 14)

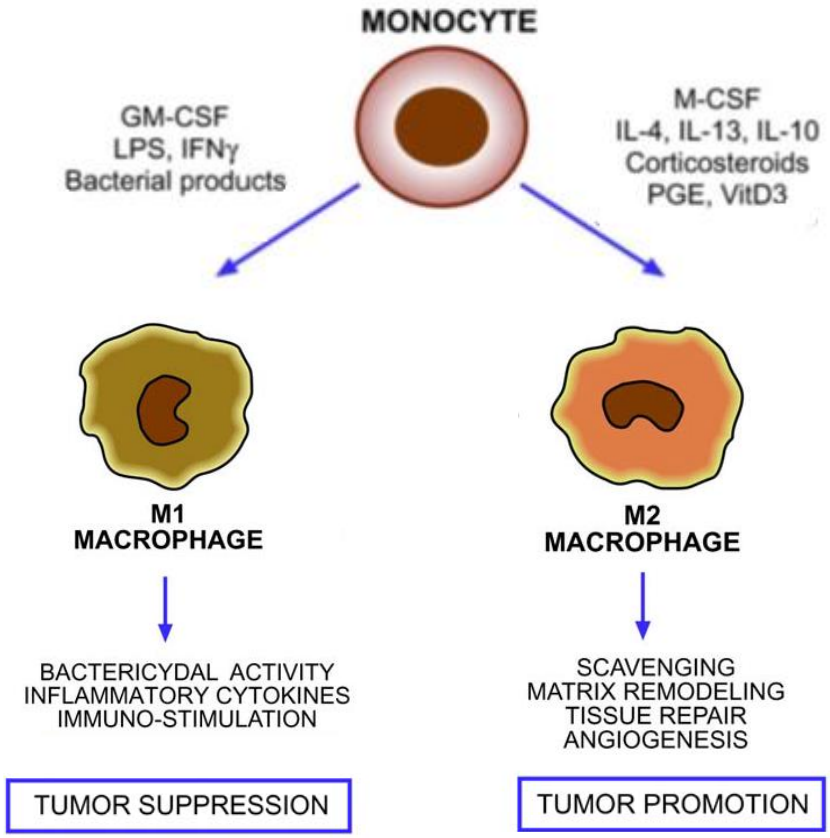

Figure 2 – Monocytes differentiation in polarized macrophages. M1 and M2 subsets diverge in terms of phenotype and functions. (Adapted from (15))

The classical or M1 macrophages are recognized as a unique program of anti-microbial response. In contrast, M2 macrophages are known for high scavenging ability, promoting tissue repair, angiogenesis and tumor progression.(15) Only recently it was recognized anti-inflammatory molecules that can induce different responses in M2 macrophage activation. These molecules are, for example IL-4 and IL-10. This distinction between M1 and M2 macrophages do not represent very well the different states of activation of macrophages but a simplified way to see the two extremes of polarization of macrophages.(15, 16)

The M1 macrophages activation occurs in response to microbial products and are characterized by a high capacity to present antigens. They produce high levels of IL-12 which consequently leads to the activation of a polarized type I response (Th1). They also produces toxic intermediates, such as nitric oxide and reactive oxygen species.(17) Thus M1 macrophages may be considered effectors cells enhancers that act against microorganisms and tumor cells, producing large amounts of pro-inflammatory cytokines. On the other hand, the response of M2 macrophages, depend on the polarized type II response (Th2), adaptive immunity. They promote angiogenesis and tissue repair and remodeling, and express low levels of IL-12 and high levels of IL-4 and IL-10. (15, 16)

The unbalance between of M1 and M2 cell number may lead to pathological events. An M1 excess may induce chronic inflammatory diseases, while an uncontrolled number of M2 may lead to severe immune suppression.(18)

Supportive to the macrophage balance hypothesis, macrophages assume different functional activation states depending on the microenvironment to which they are exposed. In the tumor microenvironment, TAMs assume an immunoregulatory M2 phenotype characterized by the expression of immunosuppressive molecules such as IL-10.(18, 19)

Tumors are mostly composed of proliferating tumor cells and stromal cells, with endothelial cells, inflammatory cells and fibroblasts. It is well-establish that TAMs plays a crucial role in tumor growth.(20) In tumor promoting biology, TAMs assume different phenotypes activating mechanisms, such as production of immunosuppressive molecules, angiogenesis and production of growth factors and proteases linked favorable to tumor invasion/metastasis.(15, 19, 21)

3.1. TAMs and Tumor Growth

As soon as monocytes reach the tumor mass, they are surrounded by several signals able to shape the cells as needed by the tumor.(18) The key factors implicated in the recruitment are chemokine CCL2, macrophage colony-stimulating factor (M-CSF) and vascular endothelial growth factor (VEGF). As they arrive the tumor, they are enclosed for several microenvironmental signals that induce the differentiation to TAMs, so they can produce the factors (IL-4, IL-10, Transforming Growth Factor beta (TGF-β)) needed by the tumor to growth.(18)

TAMs work actively for supporting tumor cell growth by producing M-CSF, VEGF, epidermal growth factor (EGF), tumor necrosis factor (TNF-α), matrix metalloproteases (MMPs) and IL-1.(18, 22, 23) All these factors contribute to the activation of a Th2 response by inhibiting the Th1 response.

Another important mechanism performed by TAMs is activation of signal transducer and activator of transcription 3 (STAT3) which in turn activates the expression of IL-6 that induce the proliferation of tumor cells.(23)

3.2. TAMs and Immunosuppression

The immunosuppressive functions of TAMs within the tumor can be activated by different ways, exhibiting factors with massive potential immunosuppressive that consequently inhibits the activity of cytotoxic T cells.(20, 24) Most studies showed that the main factors involved in this process are the TGF-β, IL-10, Arginase 1 and CCL.(20) TGF-β inhibits the activity of natural killer cells (NK) leading to a poor anti-tumor response. IL-10, a cytokine produced by TAMs, in large amounts, leading to a suppression of IL-12 and thereby inhibiting Th1 response and suppressing the activity of cytotoxic T cells.(20-22) Arginase 1 metabolizes producing polyamide and proline which causes dysregulation of the T cell receptors that induces the suppression of $CD8^+$ T. The chemokines (CCL-2) produced by TAMs induce a Th2 response, regulate the flow of T regulatory cells and suppress the response of cytotoxic T cells.(20, 21, 23)

3.3. TAMs and Angiogenesis

The process of angiogenesis involves multiple mediators and recruitment of different cell types. One of the cells involved are TAMs, which have a positively correlation with angiogenesis; increased infiltration of TAMs increased production of blood vessels leading to an increase tumor growth.(18, 22)

Epidemiological studies indicates that a full macrophage microenvironment will foster a more aggressive tumor, and consequently with a high metastatic potential.(20, 21) Tumors can't progress unless they are vascularized. It has been described that TAMs are the inducers of neovascularization and support functions.(20)

Recent publications have shown that a large number of TAMs are attracted and retained in avascular and necrotic areas when they are exposed to tumor hypoxia.(21, 25) Hypoxia is defined as a condition where the oxygen concentration is significantly lower than in healthy tissues.(26) Hypoxia is one of the most important micro environmental phenomen that control the phenotype of macrophages in pathological situations.(20, 25, 27) The effect of hypoxic areas exercised on macrophages is applied by a known transcription factor the hypoxia-inducible factor (HIF) and its two isoforms HIF-1α and HIF-2.(18, 20, 27)

There may be different hypoxic responses depending on profile of gene expression of macrophages. One of the possible changes induced by hypoxia in macrophages is the expression of cytokines and angiogenic and metastatic genes, VEGF, Platelet-derived Growth Factor (PDGF), TGF-β and MMP, as well as pro-inflammatory cytokines such as TNF-α.(18, 20, 22, 25, 27)

The process of angiogenesis is correlated with an increased macrophage infiltration by inhibition the mobility of TAMs, facilitating their accumulation in hypoxic areas.(23, 25) The number of TAMs is generally higher in tumors with high levels of hypoxia.(23) Macrophages are essential in tumor angiogenesis being performing duties in tissue repair and producing essentially VEGF in the avascular and tumor areas.(18, 28)

3.4. TAMs and Tumor Invasion and Metastasis

Studies show that primaries tumors with high counts of TAMs are correlated with the early appearance of metastasis.(22)

In the beginning, TAMs assist tumor cells in the mobility out of the mass towards the blood vessels. And then, TAMs assists cancer invasion helping tumor cells in their movement, forming clusters of tumor cells and macrophages. TAMs move alongside the tumor cells releasing EGF promoting the invasion of malignant cells.(20)

TAMs, also, release TNF-α and MMPs promoting the disruption of the basement membrane degrading the proteins of extracellular matrix.(18, 20)

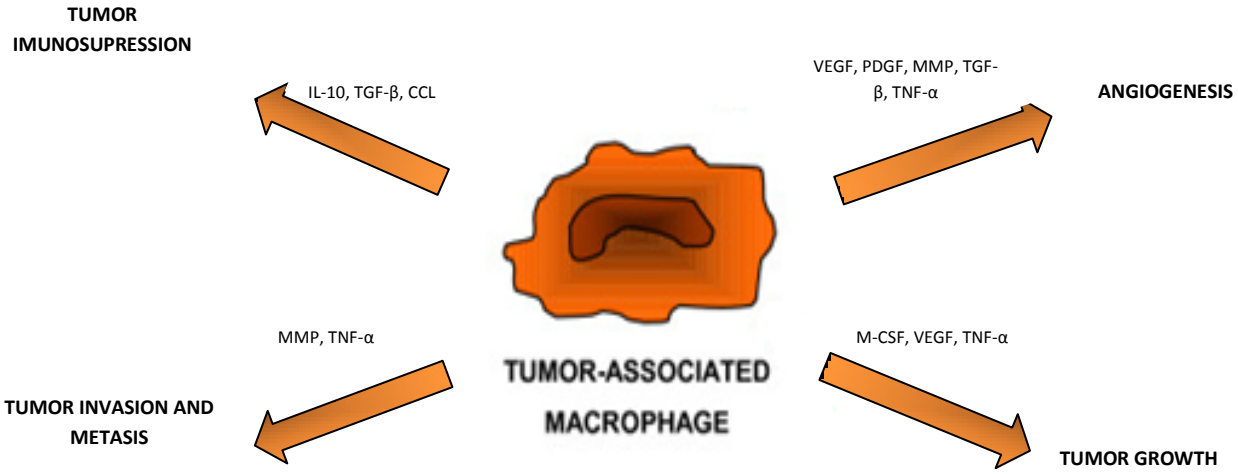

Figure 3 – A simplified vision of TAMs in the immunobiology of tumors where TAMs display some pro-tumoral functions.

4. BCG and Macrophages

The intravesical instillations of BCG induce a massive local immune response that is characterized by the expression of cytokines in urine and bladder wall and also an influx of granulocytes and mononuclear (lymphocytes and macrophages) cells. These mononuclear cells express activation markers and cellular infiltrates can persist for 12 months.(29)

After BCG being administered it can be internalized by benign cells of the urothelium or by tumor cells. After degradation, it induces the formation of antigens which are presented at the cell surface.(7, 9) In response to mycobacterial stimulation, urothelial cells secrete pro-inflammatory cytokines including IL-1, IL-6, IL-8, IL-12 and TNF-α.(17) A few hours after instillation, BCG initiates a complex inflammatory cascade of events. The first cells of innate immune response to be released consist of large numbers of neutrophils followed by monocytes/macrophages that infiltrate the bladder wall and adding characteristic cytokines and chemokines.(17) Released cytokines and chemokines attract conventional T lymphocytes, Natural Killer (NK) cells and neutrophils that cause a severe immune response leading to the neutralization of the tumor. Dendritic cells and macrophages are also capable of exercising anti-tumor effects.(9) These immune cells attract $CD4^+$T cells, leading to a T helper 1 (Th1) response.(29) Attracted immunocompetent cells form granuloma-like structures, dominated by $CD4^+$T cells, in the bladder wall.(17) Supported by the repeated BCG-instillations, formation of cellular infiltrates and granuloma-like structures proceeds for several weeks.(17)

The Th1 response, or cell immune response, and Th2 response, or humoral immune response, are responsible for different patterns cytokine secretion. Th1 response primarily secret IL-2, IL-12, Interferon-gama and TNF- α; the Th2 response secret IL-4, IL-5, IL-6 and IL-10.(11)

Qualitative analyses of the immune response indicate that the effective establishment of a Th1-cytokine profile is crucial for mounting an effective antitumor response. The importance of the Th1/Th2-dichotomy is further supported by observations that high expression levels of immunoregulatory cells like IL-10 stop Th1 responses and nullify the therapeutic effect of BCG.(17) BCG immunotherapy also promotes an innate immune response, a massive increase in the number of TAMs and lymphocytes have been reported in patient's urine after intravesical BCG therapy.(29)

More recently has been recognized that tumor biology, tumor progression and response to therapy depend on various other components of the tumor tissue, the tumor microenvironment.(19) These other components include stromal cells, infiltrating leukocytes and blood vessels (depending on tumor size), all of which contribute to the so-called tumor stroma. TAMs are a major component of the tumor stroma and experimental data suggest that TAMs may promote tumor progression.(19) TAMs may directly interfere with the BCG induced antitumor immune response and/or they may represent a marker for a tumor promoting environment.(18, 19)

At the moment, there are no suitable predictive markers of BCG treatment response. (7, 30) Recent systematic reviews highlighted that TAMs may have potential as a predictive biomarker, since when detected at tumor and surrounding tissue, it can be strongly correlated with tumor treatment response.(30) Ayari et al., in 2009, found that a higher TAM count in peri-tumoral region was associated with lower recurrence free survival and with a high risk of BCG treatment failure.(13) The same was reported for CIS tumors treated with BCG by Takayama.(14)

That is clear that an immunocompetent host response is mandatory for BCG to apply its action.(8) Therefore a superior number of TAMs could stimulate a more efficient phagocytosis and elimination of BCG, preventing BCG from inducing a long-term local inflammation and consequent anti-tumor effect. By the other hand, Takayama and coworkers(14) described that TAMs could interfere directly in BCG treatment response through the induction of immunosuppressive cytokines patterns, such as the typical pattern of cytokines produced by M2 macrophages (IL-4/IL-10).(14, 30, 31) However, it would be necessary to promote further studies in this specific issue to prove the influence of TAMs in the immune response to treatment with BCG.(30)

Macrophages are the central piece to different immune responses and are clearly immunoregulatory cells within the tumor.(21) Several studies suggest that a high number of TAMs is essential for tumor growth and progression and consequently related with poor prognosis for the patients. Although, in some studies a high number of infiltrating TAMs is correlated with better prognosis.(28)

The nature of TAMs is an essential point of study. Since it is already widely known that M2 macrophages are capable of producing factors and anti-inflammatory cytokines Th2 that could down-regulate the response to BCG treatment.(30, 31)

Furthermore, macrophages in different localizations may present different phenotypes induced by the microenvironment. For example, oxygen shortage is known to promote an accumulation of angiogenic M2-macrophages in tumor hypoxic areas, where HIF-1α enhances the expression of VEGF and decreases the production of classical Th2 cytokines.(25) Despite these observations, the influence of hypoxia in the modulation of M2-polarized macrophage distribution in bladder tumors and stroma and its association with BCG treatment outcome also remains unevaluated.

In resume, a clarification about the expression pattern of M2-macrophages in intermediate and high-risk of recurrence bladder tumors and the influence of hypoxia is needed to disclose their true predictive value in the context of BCG response. In this study we devoted to this matter by evaluating the overall TAMs ($CD68^+$) as well as the M2 phenotype, based on CD163 expression, in both stroma and tumor areas. As outlined, we correlated our findings with HIF-1α expression to disclose the influence of hypoxia in M2-macrophage accumulation and treatment outcome.

References

1. Lunt CR, Maddineni SB, Brough R. Bladder Cancer. Britist Journal of Medical and Surgical Urology. 2012(5):95-103.
2. Cross W, Whelan P. Bladder Cancer. Renal and Urology II. 2010;28(12):599-604.
3. Latini DM, Lerner SP, Wade SW, Lee DW, Quale DZ. Bladder Cancer Detection, Treatment and Outcomes: Opportunities and Challenges. Urology. 2010;75(2):334-9
4. Babjuk M, Oosterlinck W, Sylvester R, Kaasinen E, Böhle A, Palou J, et al. Guidelines on Non-muscle-invasive Bladder Cancer (TaT1 and CIS). European Association of Urology. 2012.
5. Reis LO, Ferreira U, Billis A, Cagnon VHA, Fávaro WJ. Anti-Angiogenic Effects of the Superantigen Staphylococcal Enterotoxin B and Bacillus Calmette-Guérin Immunotherapy for Nonmuscle Invasive Bladder Cancer. Urology. 2012;Vol. 187:438-45.
6. Brausi M, Witjes JA, Lamm D, Persad R, Colombel M, Buckley R, et al. Recommendations for the Management of Nonmuscle Invasive Bladder Cancer by the International Bladder Cancer Group. Urology. 2011;186:2158-67.
7. Zuiverloon TCM, Nieuweboer AJM, Ve´kony H, Kirkels WJ, Bangma CH, Zwarthoff EC. Markers Predicting Response to Bacillus Calmette-Gue´rin Immunotherapy in High-Risk Bladder Cancer Patients: A Systematic Review. European Urology. 2011:1-18.
8. Gontero P, Bohle A, Malmstrom P-U, O'Donnell MAO, Marco, Sylvester R, Witjes F. The Role of Bacillus Calmette-Gue´rin in the Treatment of Non–Muscle-Invasive Bladder Cancer. European Urology. 2010:410-29.
9. Alexandroff AB, Nicholson S, Patel PM, Jackson AM. Recent advances in bacillus Calmette–Guerin immunotherapy in bladder cancer. Future of Medicine. 2010;2(4):551-60.
10. Alexandroff A, Jackson A, O'Donnell M, James K. BCG immunotherapy of bladder cancer: 20 years on. The Lancet. 1999;353.
11. Bevers RFM, Kurth K-H, Schamhart DHJ. Role of urothelial cells in BCG immunotherapy for superficial bladder cancer. British Journal of Cancer. 2004;91:607-12.
12. Brandau S, Suttmann H. Thirty years of BCG immunotherapy for non-muscle invasive bladder cancer: A success story with room for improvement. Biomedicine & Pharmacotherapy. 2007;61:299-305.
13. Ayari C, LaRue H, Hovington H, Decobert M, Harel FB, Alain, Tetu B, et al. Bladder Tumor Infiltrating Mature Dendritic Cells and Macrophages as Predictors of Response to Bacillus Calmette-Gue´rin Immunotherapy. European Urology. 2009:1386–96.
14. Takayama H, Nishimura K, Tsujimura A, Nakai Y, Nakayama M, Aozasa K, et al. Increased Infiltration of Tumor Associated Macrophages is Associated With Poor Prognosis of Bladder Carcinoma In Situ After Intravesical Bacillus Calmette-Guerin Instillation. Urology. 2009;181:1894-900.
15. Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: Potential targets of anti-cancer therapy. European Journal of Cancer. 2006;42:717-27.

16. Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Current Opinion in Immunology. 2010;22(2):231-7.
17. Brandau S, Suttmann H. Thirty years of BCG immunotherapy for non-muscle invasive bladder cancer: A success story with room for improvement. Biomedicine&Pharmacotherapy. 2007;61:299-305.
18. Solinas G, Germano G, Mantovani A, Allavena P. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. Journal of Leukocyte Biology. 2009;86:1065-73.
19. Brandau S. Tumor Associated Macrophages: Predicting Bacillus Calmette-Guerin Immunotherapy Outcomes. The Journal of Urology. 2009;181:1532-3.
20. Hao N-B, Lü M-H, Fan Y-H, Cao Y-L, Zhang Z-R, Yang S-M. Macrophages in Tumor Microenvironments and the Progression of Tumors. J Clinical and Developmental Immunology. 2012.
21. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell. 2010;141(1):39-51.
22. Guruvayoorappan C. Tumor versus tumor-associated macrophages: how hot is the link? Integr Cancer Ther. 2008;7(2):90-5.
23. Mallmann MR, Schmidt SV, Schultze JL. Macrophages in human cancer: Current and future aspects. Atlas Genet Cytogenet Oncol Haematol. 2012;16(10).
24. Ruffell B, Affara NI, Coussens LM. Differential macrophage programming in the tumor microenvironment. Trends Immunol. 2012;33(3):119-26.
25. Murdoch C, Lewis CE. Macrophage migration and gene expression in response to tumor hypoxia. Int J Cancer. 2005;117:701–8.
26. Riboldi E, Porta C, Morlacchi S, Viola A, Mantovani A, Sica A. Hypoxia-mediated regulation of macrophage functions in pathophysiology. Int Immunol. 2013;25(2):67-75.
27. Biswas SK, Chittezhath M, Shalova IN, Lim JY. Macrophage polarization and plasticity in health and disease. Immunol Res. 2012;53(1-3):11-24.
28. Heusinkveld M, Burg SHvd. Identification and manipulation of tumor associated macrophages in human cancers. Journal of Translational Medicine. 2011;9(216).
29. BÖHLE A, BRANDAU S. Immune Mechanisms in Bacillus Calmette-Guerin Immunotherapy for Superficial Bladder Cancer. Journal of Urology. 2003;170:964-9.
30. Lima L, Dinis-Ribeiro M, Longatto-Filho A, Santos L. Predictive Biomarkers of Bacillus Calmette-Guérin Immunotherapy Response in Bladder Cancer: Where AreWe Now? Advances in Urology. 2012.
31. Van Rhijn BWG, Burger M, Lotan Y, Solsona ES, Christian G., Sylvester RJ, Witjes JA, et al. Recurrence and Progression of Disease in Non–Muscle-Invasive Bladder Cancer: From Epidemiology to Treatment Strategy. European Urology. 2009:430-42.

CHAPTER II

The predominance of M2-polarized macrophages in the stroma of low-hypoxic bladder tumors is associated with BCG immunotherapy failure

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Urologic Oncology: Seminars and Original Investigations

doi: 10.1016/j.urolonc.2013.10.012.

The predominance of M2-polarized macrophages in the stroma of low-hypoxic bladder tumors is associated with BCG immunotherapy failure

ABSTRACT

Objective: Bacillus Calmette-Guérin (BCG) immunotherapy is the gold standard treatment for superficial intermediate/high risk of recurrence or progression bladder tumors. However, approximately 30% of patients fail to respond to treatment. Effective BCG therapy needs precise activation of the Th1 immune pathway. Tumor-associated macrophages (TAMs) often assume an immunoregulatory M2 phenotype and may directly interfere with the BCG induced antitumor immune response. Thus, we aim to clarify the influence of TAMs, in particular of the M2-phenotype in stroma and tumor areas, in BCG treatment outcome.

Patients and methods: The study included 99 bladder cancer patients treated with BCG. Tumors resected prior to treatment were evaluated using immunohistochemistry for CD68 and CD163 antigens, which identify a lineage macrophage marker and a M2-polarized specific cell surface receptor, respectively. $CD68^+$ and $CD163^+$ macrophages were evaluated within the stroma and tumor areas and high density of infiltrating cells spots were selected for counting. Hypoxia, an event known to modulate macrophage phenotype, was also accessed through HIF-1α expression.

Results: Patients in which BCG failed had high stroma-predominant $CD163^+$ macrophage counts (high stroma but low tumor $CD163^+$ macrophages counts) when compared with the ones with a successful treatment (71% vs. 47%; p=0.017). Furthermore, patients presenting this phenotype showed decreased recurrence-free survival (log rank, p=0.008) and a clear 2-fold increased risk of BCG treatment failure was observed in univariate analysis (HR=2.343; 95%CI: [1.197-4.587]; p=0,013). Even when adjusted to potential confounders, such as age and therapeutic scheme, multivariate analysis revealed 2.6-fold increased risk of recurrence (HR=2.627; 95%CI: [1.340-5.150]; p=0.005). High stroma-predominant $CD163^+$ macrophage counts were also associated with low expression of HIF-1α in tumor areas, whereas high counts of $CD163^+$ in the tumor, presented high expression of HIF-1α in tumor nests.

Conclusions: TAMs evaluation using CD163 is a good indicator of BCG treatment failure. Moreover, elevated infiltration of $CD163^{+}$ macrophages predominantly in stroma areas but not in the tumor may be a useful indicator of BCG treatment outcome, possibly due to its immunosuppressive phenotype.

Keywords: Bladder cancer, BCG immunotherapy, Tumor-associated macrophages, CD68, CD163

INTRODUCTION

Bladder cancer (BC) is the second most common urologic cancer and has the highest recurrence ratio of any malignancy. (1) Approximately 75-85% of all BC are non-muscle invasive (NMIBC), which includes carcinoma in situ (CIS) and papillary tumors confined to the mucosa or submucosa (Ta/T1). (1) The NMIBC risk classification divides patients into low, intermediate and high-risk categories for recurrence and progression. (1) The gold standard treatment for intermediate/high risk patients is intravesical instillations with Bacillus Calmette-Guérin (BCG). (1) However, 30–50% of patients fail to respond, and 15% show progression to muscle-invasive disease. In these cases, radical cystectomy is the treatment to follow.(2) Intravesical instillations of BCG induce a massive local immune response that is characterized by the expression of cytokines in the bladder, as well as an influx of granulocytes and mononuclear cells (lymphocytes and macrophages) into the tumor areas.(3, 4)

Tumor biology, tumor progression and response to therapy are influence by the tumor microenvironment.(5, 6) These includes stromal cells, infiltrating leukocytes and blood vessels (depending on tumor size), all of which contribute to the so-called tumor stroma.(6) Tumor-associated macrophages (TAMs) are a major component of the tumor stroma that contributes to tumor progression in several types of cancer.(6, 7)

Macrophages are polarized in two distinct functional forms, M1 and M2.(6-8) The classical or M1 macrophages activate type 1 helper cells (Th1) that have the capability to kill pathogens, produce IL-2, IL-12 and pro-inflammatory cytokines that promote responses like cytotoxic T-cell activation.(8) In contrast, alternatively-activated M2 macrophages express low levels of IL-12 and high levels of IL-4 and IL-10, promoting Th2 cytokines that inhibit Th1 responses.(7) However, associated to the tumor, M2-polarized macrophages comprise multiple subtypes that may contribute to immunosuppression, angiogenesis, cell invasion and metastasis, depending on the microenvironment.(5, 9) Also, cytokines and chemokines released by the tumor may recruit and modulate monocyte differentiation into M2-macrophages lineages that may differ from those in the stroma.(6, 7) As such, a detailed evaluation of macrophage phenotypes in both the tumor and the stroma as well as their microenvironment is needed to fully understand how M2-macrophages influence tumor behavior and ultimately the response to treatment.

The studies presented so far indicate that higher TAM counts are associated with lower recurrence free survival and high risk of BCG treatment failure.(10-12) Nevertheless, these conclusions were based exclusively on CD68 expression, a macrophage lineage marker that does not allow the discrimination between M1 and M2 phenotypes therefore adding bias to these observations.(13, 14) Also, patients that respond to BCG commonly release large amounts of Th1 cytokines (15), whereas high levels of Th2 cytokines (*i.e.* IL-4 and IL-10) seem to be related with BCG failure.(16) These observations support the idea that effective BCG therapy requires precise activation of the Th1 immune pathway.(17, 18) However, TAMs assuming an immunoregulatory M2-phenotype release Th2 cytokines that may directly interfere with the BCG induced antitumor immune response.(7, 16, 18) Still, no direct evidences have been presented supporting the association between higher counts of M2-polarized macrophages and the failure of BCG treatment.

Furthermore, macrophages in different localizations may present different phenotypes induced by the microenvironment. For example, oxygen shortage is known to promote an accumulation of angiogenic M2-macrophages in tumor hypoxic areas, where HIF-1α enhances the expression of VEGF and decreases the production of classical Th2 cytokines.(19) Despite these observations, the influence of hypoxia in the modulation of M2-polarized macrophage distribution in bladder tumors and stroma and its association with BCG treatment outcome also remains unevaluated.

In resume, a clarification about the expression pattern of M2-macrophages in intermediate and high-risk of recurrence bladder tumors and the influence of hypoxia is needed to disclose their true predictive value in the context of BCG response. In this study we devoted to this matter by evaluating the overall TAMs ($CD68^+$) as well as the M2 phenotype, based on CD163 expression, in both stroma and tumor areas. As outlined, we correlated our findings with HIF-1α expression to disclose the influence of hypoxia in M2-macrophage accumulation and treatment outcome.

METHODS

Cohort of Patients

In this study were included 99 formalin fixed paraffin embedded (FFPE) tissues from patients treated with transurethral resection of bladder tumor (TURBT) and then submitted to BCG immunotherapy in the Urology Department of Portuguese Institute of Oncology – Oporto (IPO-Porto), between 1998 and 2006. All patients received induction BCG therapy for 6 consecutive weeks, starting 2-3 weeks after surgery (iBCG) and were then submitted to mBCG schedule (the one used in our institute is iBCG + maintenance protocol with 2-weekly instillations every 3 months during 2 years). The iBCG group includes patients treated before the European Association of Urology guidelines recommending the mBCG scheme [20] and patients showing significant intolerance to long BCG treatment.

The average age of the patients was 68 years (range 41-85). The male:female ratio was 84:15. The patients were followed every 3 months during the first year, every 6 months in the second year and every 12 months thereafter by cystoscopy and urine cytology. The median follow-up time was 68 months (range:10-163months).

Recurrence was defined as the appearance of a tumor after the beginning of the treatment, with at least one tumor-free cystoscopy and cytology in-between. BCG failure, as opposed BCG success, was defined as patients submitted to BCG treatment with tumor recurrence. Finally, recurrence-free survival (RFS) was defined as the period between the beginning of the treatment and either recurrence or the most recent tumour-free cystoscopy and cytology. All procedures were performed after patient's informed consent and approved by the Ethics Committee of IPO-Porto. All clinicopathological information was obtained from patients' clinical records. All tumour samples were revised by an experienced pathologist, regarding 2004 WHO grading criteria.

Immunohistochemistry

TAMs immunohistochemistry was performed with CD68 antibody (Monoclonal Mouse Anti-Human CD68; Clone PG-M1; DAKO) at a dilution of 1:100 in PBS, after 1h incubation at 37°C. M2 macrophages were accessed with the CD163 antibody (Monoclonal Mouse Anti-Human CD163; Clone 10D6; Novocastra-Leica) at a dilution of 1:100 in PBS, after overnight incubation at 37°C. Immunohistochemical detections were performed using HRP Detection System Kit according to manufacturer's instructions. Diaminobenzidine (Impact Dab, Vector Labs) was used for color development. Hypoxic sites were evaluated using HIF-1α antibody (Monoclonal Mouse Anti-Human HIF-1α; Clone H1α67; Abcam) at a dilution of 1:50 in PBS, after overnight incubation at 37°C.

Immunohistochemistry Scoring

$CD68^+$ and $CD163^+$ macrophages, infiltrating the stroma and tumor areas were counted by two independent observers (LL, DO) and validated by an experienced pathologist (TA). Each specimen was screened at low magnification (100x) and the five areas with highest number of positively stained cells (hot-spot area) were selected. Photographs were taken, at a 400x magnification, with a real area of 0.035 mm^2, and TAMs number was counted. The criteria used for macrophage specific counting were as follows: i) cells must present the shape of a macrophage and exhibit the macrophage characteristic staining pattern; ii) must present cell nucleus; and iii) be birefringent if the size is small;. Macrophages were evaluated in the tumor stroma, which included the papillary axis, lymphoid aggregates and stroma, and in tumor islets. Macrophages counts were classified as low or high according to their distribution in percentiles. The expression of HIF-1α was determined based on percentage of positive cells and stratified in groups was as follows: Low (negative or 1-10% nuclear or cytoplasmic staining) and High (10-50% or >50% nuclear or cytoplasmic staining).

Statistical Analysis

Statistical data analysis was performed using the IBM Statistical Package for Social Sciences—SPSS for Windows (version 20.0). Chi-square analysis was used to compare categorical variables. Correlation between macrophage counts and clinical variables was performed using Spearman rho test. Kaplan-Meier survival curves were used to evaluate correlation between TAMs counts and RFS, log-rank statistical test was used for curves comparison. Multivariate Cox regression analysis was used to assess the effect of TAMs density on the time to recurrence in BCG-treated patients and to adjust for potential confounders.

RESULTS

Association of Clinical and clinical characteristics with BCG treatment outcome

Approximately 42.4% of the patients presented recurrences, with the median recurrence time of 38.5 months (range: 10-122). The median follow-up time of the patients free of recurrence was 97 months (range: 13-163).

Table 1 shows the clinicopathologic parameters and its association with treatment response and RFS. An association was found between patients' age and treatment response, since 69% of the patients presenting BCG failure were over 65 years when compared with 43.9% in the BCG success group (p=0.013). Consequently, patients over 65 years presented almost a 3-fold increased risk of recurrence (HR=2.763; 95%CI: [1.431-5.336]; p=0.002). Similarly, patients treated with mBCG presented a 50% risk reduction of recurrence (HR=0.500; 95%CI: [0.271-0.919]; p=0.026). Approximately 70% of the patients successfully treated were submitted to mBCG scheme (*vs.* 45% of the patients presenting treatment failure, p=0.021). Interestingly, no association was found between treatment outcome and other characteristics such as gender, tumour stage, number, grade or size, CIS presence and prior recurrence.

Pattern of macrophage infiltration

We started by evaluating the localization of macrophages within tumor specimens. We observed the presence of $CD68^+$ and $CD163^+$ macrophages in both tumor stroma and in tumor islets. The tumor stroma included the papillary axis, lymphoid aggregates and stroma. The mean count of $CD68^+$ macrophages was 33 within stroma and 13 within tumor while for $CD163^+$ macrophages within stroma and tumor was, respectively, 24 and 7. The mean ratio of $CD163^+/CD68^+$ macrophages was 51.3% in the tumor and 24.6% in the associated stroma. A moderate to strong $CD68^+$ macrophage stroma infiltration was observed in 46% of the tumors while only 4% of cases had none $CD68^+$ macrophage staining in tumor nest. A high $CD163^+$ macrophages stroma infiltration was observed in 15% tumors while only 8% of tumors presented no CD163 staining in tumor nests.

Table 1 – Relation between patients clinical and tumour characteristics and response to BCG treatment and time to recurrence.

Variables	Total	Responders	Non-Responders	HR [95% CI]	p*
	n (%)	n (%)	n (%)		
Age					
<65 years	45 (45.5)	32 (56.1)	13 (31.0)	1.0	
≥65 years	54 (54.5)	25 (43.9)	29 (69.0)	2.763 [1.431-5.336]	**0.002**
Sex					
Male	84 (84.8)	46 (80.7)	38 (90.5)	1.0	
Female	15 (15.2)	11 (19.3)	4 (9.5)	0.526 [0.187-1.478]	0.223
Stage					
Ta	40 (40.4)	22 (38.6)	18 (42.9)	1.0	
T1	59 (59.6)	35 (61.4)	24 (57.1)	0.961 [0.521-1.773]	0.899
Grade					
Low	39 (39.4)	24 (42.1)	15 (35.7)	1.0	
High	60 (60.6)	33 (57.9)	27 (64.3)	1.410 [0.749-2.654]	0.287
Size (cm)					
<3	64 (65.3)	36 (63.2)	28 (68.3)	1.0	
≥3	34 (34.7)	21 (36.8)	13 (31.7)	0.760 [0.393-1.470]	0.416
Tumor number					
Unifocal	45 (45.5)	29 (50.9)	16 (38.1)	1.0	
Multifocal	54 (54.5)	28 (49.1)	26 (61.9)	1.729 [0.924-3.235]	0.087
CIS					
No	92 (92.9)	53 (93.0)	39 (92.9)	1.0	
Yes	7 (7.1)	4 (7.0)	3 (7.1)	0.944 [0.291-3.056]	0.923
Recurrence Status					
Primary	51 (51.5)	33 (57.9)	18 (42.9)	1.0	
Recurrent	48 (48.5)	24 (42.1)	24 (57.1)	1.562 [0.847-2.881]	0.153
BCG schedule					
iBCG	41 (41.4)	18 (31.6)	23 (54.8)	1.0	
mBCG	58 (58.6)	39 (68.4)	19 (45.2)	0.500 [0.271-0.919]	**0.026**

HR: Hazard Ratio; CI: Confidence Interval; CIS: Carcinoma *in situ.*

*: Wald test

Correlation between clinical characteristics and $CD68^+$ and $CD163^+$ macrophage counts

The correlation between clinical variables and the macrophages counts are indicated in Table 2. Our results evidenced that $CD68^+$ and $CD163^+$ macrophages counts within stroma and tumor were correlated with higher stage, grade and tumor size (Table 2, $p<0.05$). Similarly, higher counts of CD68 and CD163 within tumor were observed in primary tumors (Table 2, $p<0.05$). Interestingly, higher CD163/CD68 ratios in tumor nests were associated with the CIS presence (Table 2, $p <0.05$). No correlations were found regarding gender, age and multifocality.

Table 2 – Correlation between clinical parameters and $CD68^+$ and $CD163^+$ macrophages counts, in tumor stroma and tumor nest.

	$CD68^+$ macrophages counts				**$CD163^+$ macrophages counts**				**$CD163^+/CD68^+$ macrophage ratio**			
	Tumor Stroma		**Tumor Nest**		**Tumor Stroma**		**Tumor Nest**		**Tumor Stroma**		**Tumor Nest**	
	Correlation coefficient	*P* value	Correlation Coefficient	*P* value	Correlation Coefficient	*P* value	Correlation Coefficient	*P* value	Correlation Coefficient	*P* value	Correlation Coefficient	*P* value
Age	0.026	0.802	-0.030	0.770	0.012	0.906	0.034	0.735	-0.015	0.884	0.014	0.897
Sex	-0.046	0.649	0.129	0.204	-0.066	0.519	0.166	0.101	-0.028	0.786	0.074	0.477
Stage	0.371	**0.000**	0.271	**0.007**	0.274	**0.006**	0.300	**0.003**	0.116	0.253	-0.012	0.907
Grade	0.284	**0.004**	0.194	0.054	0.232	**0.021**	0.278	**0.005**	0.135	0.184	0.126	0.223
Size	0.263	**0.009**	0.290	**0.004**	0.172	0.090	0.284	**0.005**	0.220	**0.029**	0.109	0.294
Tumor Number	-0.081	0.424	-0.023	0.818	0.004	0.972	0.012	0.903	-0.062	0.544	0.093	0.369
CIS	-0.176	0.082	0.072	0.481	-0.137	0.178	0.046	0.655	0.105	0.302	0.280	**0.006**
Primary / Recurrent	-0.120	0.239	-0.273	**0.006**	-0.037	0.718	-0.229	**0.023**	0.023	0.824	0.020	0.850

CIS: Carcinoma *in situ.*
P value : Chi-square test

CD68$^+$ and CD163$^+$ macrophages and BCG treatment outcome

To evaluate the CD68$^+$ and CD163$^+$ macrophages infiltration within stroma and tumor areas in the context of BCG treatment outcome, counts were stratified based on percentiles (25th,50th,75th). The same strategy was applied for the CD163/CD68 ratio. (Table 3)

Regarding CD68 expression, no association was found between the counts and treatment outcome. On the other hand we observed that only CD163$^+$ stroma counts falling within the 25th percentile (>19 macrophages) presented a trend association with treatment outcome; CD163$^+$ macrophage counts in the stroma were classified as low (LS) or high (HS) accordingly. Namely, a higher frequency of patients with BCG failure presented HS (above the 25th percentile) for CD163$^+$macrophages (83%) when compared with ones where BCG was successful (74%); yet this association was not statistically significant.

We also observed that the LS phenotype was always associated with low macrophage tumor counts (LT) (Fig. 4A). Furthermore, the CD163$^+$ LS phenotype (associated with LT) presented BCG treatment response rates similar to the cases with HS and high tumor CD163$^+$counts (HT; >75th percentile – Fig. 4B). Based on these observations, we decided to merge these two groups (LS/LT and HS/HT) and compare it with the cases presenting HS but LT CD163$^+$ counts. Taking into consideration the low CD163$^+$ counts presented by the tumors included in the LT phenotype (<10 macrophages) in comparison with the high stroma counts, the group was termed high stroma-predominant CD163$^+$ macrophage group (HSP, Fig. 4C).

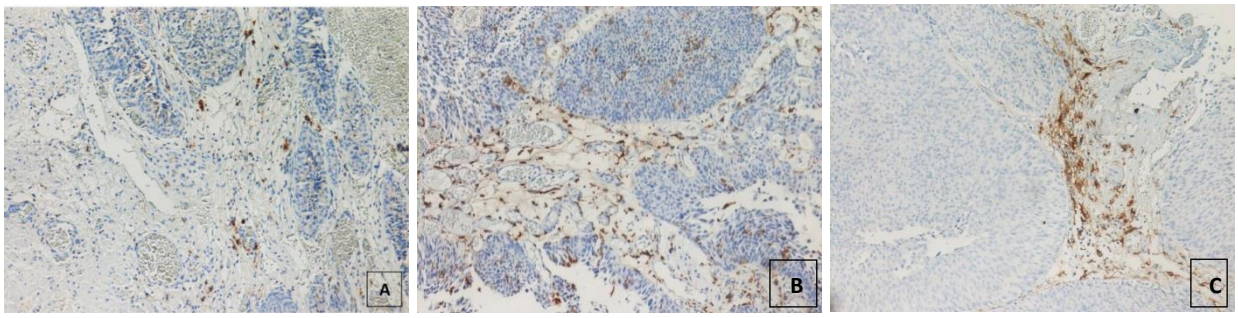

Figure 4 – Immunohistochemical staining showing different grades of CD163$^+$ macrophages infiltration in bladder tumors (400x). Representative images (A-C) of macrophages stained with anti-CD163 (brown). A- Low Stroma and Low Tumor Macrophages infiltration (LS/LT); B- High Stroma and High Tumor Macrophages infiltration (HS/HT); C- High Stroma and Low Tumor Macrophages infiltration (HS/LT) – High Stroma-predominant Macrophages Counts.

Table 3 – Relation between the counts of positive macrophages and patients

Variables	Total	Responders	Non-Responders	p*	HR [95% CI]	p**
	n (%)	n (%)	n (%)			
CD68 Stromal						
≤27.8 (25th percentile)	25 (25.3)	17 (29.8)	8 (19.0)	0.223		
>27.8 (25th percentile)	74 (74.7)	40 (70.2)	34 (81.0)		1.875 [0.865-4.064]	0.111
≤33.4 (50th percentile)	50 (50.5)	29 (50.9)	21 (50.0)	0.931		
>33.4 (50th percentile)	49 (49.5)	28 (49.1)	21 (50.0)		1.219 [0.662-2.242]	0.525
≤38.4 (75th percentile)	75 (75.8)	42 (73.7)	33 (78.6)	0.575		
>38.4 (75th percentile)	24 (24.2)	15 (26.3)	9 (21.4)		1.209 [0.606-2.412]	0.589
CD68 Tumor						
≤7.2 (25th percentile)	25 (25.3)	14 (24.6)	11 (26.2)	0.854		
>7.2 (25th percentile)	74 (74.7)	43 (75.4)	31 (73.8)		1.461 [0.689-3.098]	0.323
≤11.4 (50th percentile)	51 (51.5)	28 (49.1)	23 (54.8)	0.579		
>11.4 (50th percentile)	48 (48.5)	29 (50.9)	19 (45.2)		1.029 [0.558-1.895]	0.928
≤16.4 (75th percentile)	75 (75.8)	41 (71.9)	34 (81.0)	0.301		
>16.4 (75th percentile)	24 (24.2)	16 (28.1)	8 (19.0)		0.842 [0.389-1.822]	0.663
CD163 Stromal						
≤19 (25th percentile)	25 (25.3)	17 (29.8)	8 (19.0)	0.223		
>19 (25th percentile)	74 (74.7)	40 (70.2)	34 (81.0)		2.115 [0.972-4.603]	**0.059**
≤25.2 (50th percentile)	50 (50.5)	29 (50.9)	21 (50.0)	0.931		
>25.2 (50th percentile)	49 (49.5)	28 (49.1)	21 (50.0)		1.248 [0.680-2.291]	0.475
≤28.8 (75th percentile)	75 (75.8)	41 (71.9)	34 (81.0)	0.301		
>28.8 (75th percentile)	24 (24.2)	16 (28.1)	8 (19.0)		0.828 [0.382-1.793]	0.631
CD163 Tumor						
≤2 (25th percentile)	25 (25.3)	13 (22.8)	12 (28.6)	0.514		
>2 (25th percentile)	74 (74.7)	44 (77.2)	30 (71.4)		1.018 [0.520-1.994]	0.958
≤5.8 (50th percentile)	50 (50.5)	29 (50.9)	21 (50.0)	0.931		
>5.8 (50th percentile)	49 (49.5)	28 (49.1)	21 (50.0)		1.326 [0.719-2.444]	0.366
≤10 (75th percentile)	75 (75.8)	41 (71.9)	34 (81.0)	0.301		
>10 (75th percentile)	24 (24.2)	16 (28.1)	8 (19.0)		0.890 [0.411-1.927]	0.767
CD163/CD68 Stromal						
≤40.95 (25th percentile)	25 (25.3)	14 (24.6)	11 (26.2)	0.854		
>40.95 (25th percentile)	74 (74.7)	43 (75.4)	31 (73.8)		1.285 [0.630-2.619]	0.491
≤54.54 (50th percentile)	50 (50.5)	30 (52.6)	20 (47.6)	0.622		
>54.54 (50th percentile)	49 (49.5)	27 (47.4)	22 (52.4)		1.229 [0.667-2.265]	0.509
≤62.1 (75th percentile)	75 (75.8)	41 (71.9)	34 (81.0)	0.301		
>62.1 (75th percentile)	24 (24.2)	16 (28.1)	8 (19.0)		0.789 [0.364-1.711]	0.549
CD163/CD68 Tumor						
≤5 (25th percentile)	28 (28.3)	18 (31.6)	10 (23.8)	0.396		
>5 (25th percentile)	71 (71.7)	39 (68.4)	32 (76.2)		1.567 [0.766-3.203]	0.218
≤18.8 (50th percentile)	52 (52.5)	31 (54.4)	21 (50.0)	0.666		
>18.8 (50th percentile)	47 (47.5)	26 (45.6)	21 (50.0)		1.318 [0.714-2.433]	0.378
≤39.1 (75th percentile)	76 (76.8)	43 (75.4)	33 (78.6)	0.715		
>39.1 (75th percentile)	23 (23.2)	14 (24.6)	9 (21.4)		1.026 [0.490-2.148]	0.947

HR: Hazard Ratio; CI: Confidence Interval

*: Chi-square test; **: Wald test

This comparison highlighted that a higher percentage of patients presenting BCG failure had HSP when compared to the ones where the treatment was successful (69% vs. 46%; p=0.020; Sensivity: 54.4%; Specifity: 69.1%; Fig. 5). No association was found regarding $CD163^+/CD68^+$ ratio and BCG treatment outcome.

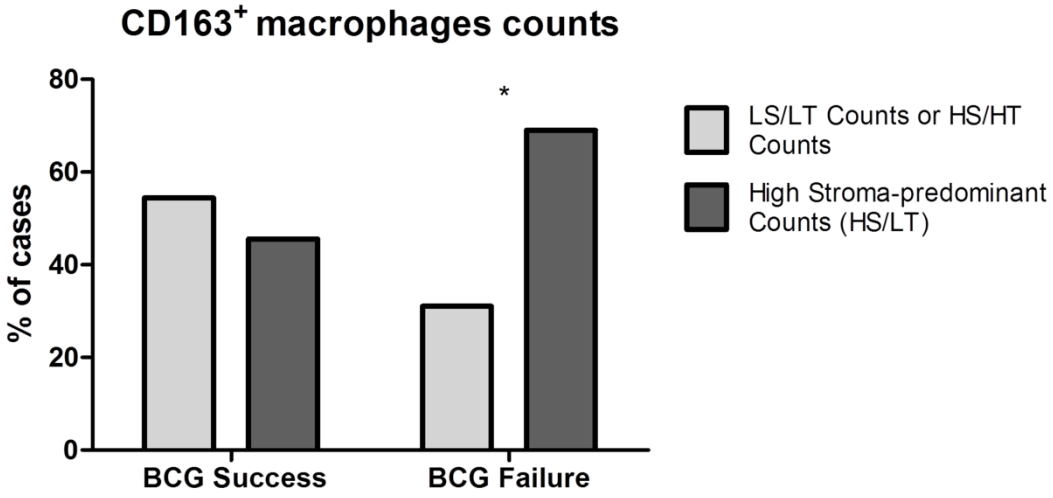

Figure 5 – Association between combined $CD163^+$ macrophages counts and BCG treatment failure. Higher stroma-restricted $CD163^+$ macrophages counts were associated with non-response after BCG immunotherapy. LS/LT: Low Stromal and Low tumor; HS/HT: High Stromal and High Tumor; HS/LT: High Stromal and Low Tumor – High Stroma-predominant Counts. "*" p=0.020 (Chi-square Test).

In order to estimate the influence of higher $CD163^+$ macrophages counts in terms of RFS after BCG treatment, a Kaplan-Meier analysis was performed (Fig. 6). Differences were found in terms of RFS between patients with LS and HS counts of $CD163^+$ macrophages (mean RFS: 126 vs. 92 months; log rank, p=0.052; Fig.6A). Moreover, patients with HSP $CD163^+$ macrophages counts presented a different behavior in terms of RFS (log rank, p=0.008; Fig.6B) and a lower RFS (mean: 85 months) than all the others (mean:123 months).

Univariate Cox Regression analysis revealed that patients with tumors presenting $CD163^+$ macrophages HS counts had an increased risk trend for recurrence after treatment, (HR=2.115; 95%CI: [0.972-4.603]; p=0.059). Moreover, patients with tumors classified as HSP showed a clear 2-fold increased risk of BCG treatment failure (HR=2.343 95%CI: [1.197-4.587] p=0,013).

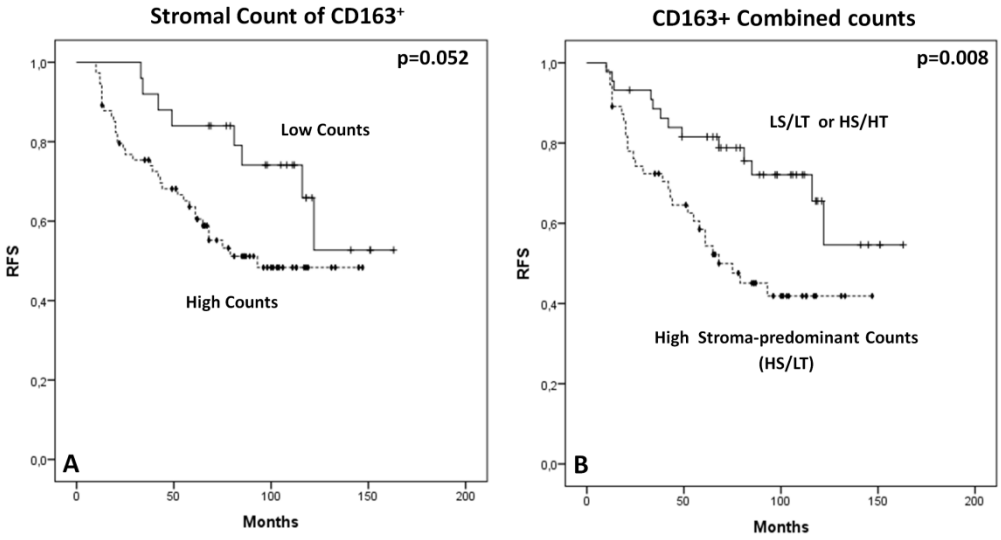

Figure 6 – Effect M2-polarized TAMs in recurrence-free survival (RFS). Kaplan-Meier analysis to evaluate the association between RFS in the studied patients and: A- $CD163^+$ macrophages counts in stroma; B- $CD163^+$ macrophages combined counts. High Stroma-predominant counts (HS/LT: High Stromal but Low Tumor Counts) *vs.* LS/LT (Low Stromal and Low Tumor counts) or HT/HT (High Stromal with High Tumor counts). Comparison performed by log-rank test (A: p=0.052; B: p=0.008); + censored "Low Counts" or "LS/LT or HS/HT" cases; ♦ censored "High Counts" or "High Stroma-predominant Counts (HS/LT)" cases.

To assess the individual effect of $CD163^+$ macrophage infiltration in BCG treatment outcome, multivariate analysis was performed. When adjusted to potential confounders, such as age and therapeutic scheme, patients with HS $CD163^+$ and HSP $CD163^+$ counts had more than a 2-fold increased risk of recurrence (respectively, HR=2.402 95%CI: [1.211-4.763] p=0.012 and HR=2.627 95%CI: [1.340-5.150] p=0.005; Table 4).

Table 4 – Multivariate analysis and risk estimation of $CD163^+$ macrophages influence on BCG therapy outcome.

$CD163^+$ macrophages	**HR[a]**	**95%CI**	**p value**
Stromal Counts			
Low (≤19)	1.0	Referent	
High (>19)	2.402	1.211-4.763	0.012
Combined Counts			
LS/LT or HS/HT	1.0	Referent	
High Stroma-predominant Counts (HS/LT)	2.627	1.340-5.150	0.005

LS/LT: Low Stromal and Low tumor counts;
HS/HT: High Stromal and High Tumor counts ;
HS/LT: High Stromal but Low Tumor counts;
HR: Hazard Ratio; CI: Confidence Interval
[a] adjusted for age and BCG schedule

<u>$CD163^+$ Macrophages and expression of HIF-1α</u>

The association between $CD163^+$ macrophages counts within tumor and hypoxia was evaluated based on HIF-1α expression. The expression of HIF-1α is represented by a nuclear and cytoplasmic staining at the invasive front of the tumor. It was also observed that tumor areas with high density of $CD163^+$ macrophages expressed high amounts of HIF-1α (Fig. 7A&B). On the other hand, tumor areas with low $CD163^+$ macrophages counts, independently of the counts in the stroma, presented lower expression of HIF-1α (Fig. 7C&D). In resume, while the HSP and LS (that also present LT counts) phenotypes were associated with tumors showing low degree of hypoxia, samples presenting the HS/HT phenotypes are associated with highly hypoxic tumors ($p<0.001$). However, the expression of HIF-1α was not associated with BCG treatment outcome.

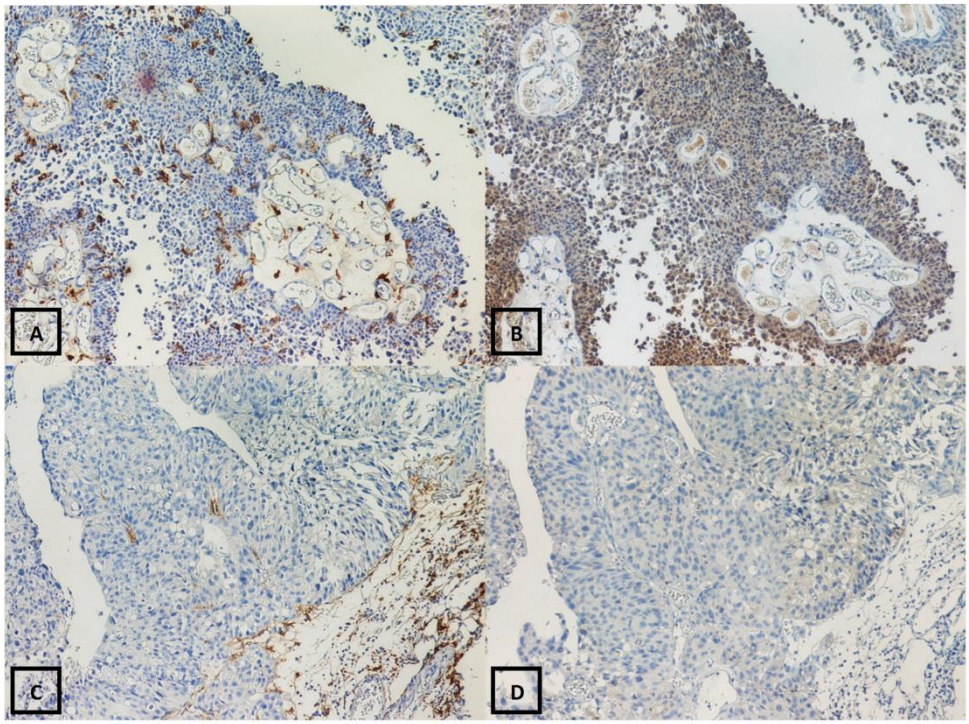

Figure 7 – Immunohistochemical staining showing different grades of $CD163^{+}$ macrophages infiltration and HIF-1α in the tumor nest of bladder cancer (100x). Representative images (A and C) of Macrophages stained with anti-CD163 (brown) and the same areas stained with anti-HIF-1α (brown - B and D). A- High infiltration of $CD163^{+}$ Macrophages in tumor; B- High staining of HIF-1 α in tumor; C- Low infiltration of $CD163^{+}$ Macrophages in tumor; D- Low staining of HIF-1 α in tumor;

Discussion

Although BCG immunotherapy is the primary treatment option for intermediate/high risk bladder tumors, the failure rate is over 30%.(1) Therefore the identification of biomarkers able to predict treatment failure and to provide an early identification of those patients better served by alternative therapies is crucial for the management of this disease.(4) There are some biomarkers emerging in the literature, but at the moment none could be set as reliable to translate into clinical practice.(21, 22)

One of the biomarkers with consistent results was the presence of TAMs in bladder tumors prior to BCG treatment, although more studies are needed to validate its relevance. (10-12, 21, 22) On the other hand, the marker used was CD68, a lineage marker found in both M1 and M2 macrophages.(7) Several authors showed that in order to accurately determine TAMs influence in prognosis and treatment outcome, M2-specific markers, such as CD163, should be used.(23-26) To address this subject we investigated the influence of TAMs ($CD68^+$) and also the M2-polarized macrophage phenotype ($CD163^+$), in the context of BCG treatment outcome. Taking into consideration that the microenvironment plays a determinant role in the modulation of the macrophage lineages we evaluated independently the tumor and the stroma.

We started by seeking associations between the patient's clinicopathological characteristics and the BCG treatment outcome and found that it was influenced by age and treatment scheme (iBCG, mBCG). Therefore, these variables were considered potential confounders and were taken into account in multivariate analysis models to assess the TAMs influence in BCG outcome. We also observed that $CD68^+$ and $CD163^+$ macrophages counts in both the stroma and tumor were correlated with higher stage, grade and tumor size. Similar results were observed by other authors for bladder cancer using CD68.(12, 27) The $CD163^+$ macrophages identification has also been associated with poor prognosis in several types of cancer (23, 28); however this is the first study suggesting that the M2-subtype may be a characteristic of high-risk of recurrence/progression bladder tumors.

Three studies have been presented supporting the idea that a higher density of macrophages in the tumor and its surroundings may be associated with BCG treatment failure.(10-12) However, we observed no associations between $CD68^+$ macrophage counts in stroma and in tumor nests and the outcome. Even though contradictory these results may stem from the fact that two of this studies were conducted in a low number of samples (27 and 46) and did not take into consideration the localization of the macrophages. A third study involving a localization-based analysis in CIS, described that cases with a low density of tumor $CD68^+$ macrophages presented higher recurrence-free rate. However the reduced number of CIS in our series does not allow an accurate comparison. Nevertheless, whether macrophage density influences treatment outcome in different ways depending on the histology of the tumor warrants a deeper evaluation.

Contrastingly, we observed that a high density of M2-polarized macrophage counts in the stroma but not in the tumor related with BCG treatment failure. Interestingly, cases presenting a high density of macrophages in the tumor presented a more favourable outcome. Furthermore, these cases behaved similarly to those presenting an overall low density of M2 macrophages (LT/LS). These results suggest that M2-macrophages may be influencing treatment outcome in different ways possibly due to the influence of differentiated micro environmental stimuli in the stroma and the tumor.

Since TAMs may be found in vascularised stroma but also significantly accumulate in hypoxic areas within the tumor [19, 29, 30], we hypothesized that differences in CD163 expression between tumor areas could be the result of hypoxia. This was confirmed by the association between high tumor $CD163^+$ macrophage counts and high expression of the hypoxia marker HIF-1α within tumor areas; conversely, in specimens with High stroma-predominant $CD163^+$ counts (and respectively low tumor counts), HIF-1α expression within tumor areas was low. These observations suggest that hypoxic conditions may dictate the accumulation of $CD163^+$ macrophages in bladder tumor areas.

Hypoxia not only seems to dictate the accumulation of macrophages in the tumor but may also modulate the M2-macrophage phenotype. In particular, hypoxia is known to enhance the expression of angiogenic factors, producing high amounts of VEGF and other proinflammatory cytokines like TNF-α, IL-1β, MIF and COX2 that act as promoters of a Th1 mediated response known to favour BCG action [19]. On the other hand, normoxia may favour the M2 immunosuppressive phenotype and the downregulation of molecules

implicated in immunological activation such as IL-12, IL-18, IL-1β and TNFα [5]. This selective pressure also upregulates the expression of Th2-type cytokines, as well as IL-10, IL-1RA and TGF-β, some of which have been associated with a lack of response to BCG treatment [16]. Based on these observations we hypothesize that hypoxic conditions may favour the accumulation of M2-polarized macrophages in the tumor and also promote their angiogenic phenotype, ultimately leading to a better treatment outcome. Conversely, non-hypoxic or low-hypoxic conditions (low HIF-1α) decrease the density of macrophages in the tumor area, maintaining them in the stroma area. We may hypothesize that these macrophages present the immunosuppressive phenotype, which in part may explain the higher treatment failure.

Although our results point out that high stroma-predominant $CD163^{+}$ macrophage counts is a good predictor of recurrence after BCG treatment, some limitations need to be overcome in order to use this biomarker in clinical practice. Namely, efforts should be taken to make the macrophage counts reproductive. It would be important to evaluate different counting methodologies, especially involving image acquisition and automatic counting software in order to create a standard technique and cut-off values. Also, this a preliminary study with 99 patients that requires validation in larger series and different cohorts. A careful evaluation of the influence of hypoxia and other microenvironment factors in the modulation of macrophage phenotypes is also needed in this context.

Altogether, our results indicate that discrimination of M2 macrophages ($CD163^{+}$) is a better indicator of treatment failure than the overall macrophage counting given by CD68. Moreover, our observations suggest that only M2 macrophages under normoxic conditions may exert an inhibitory effect on BCG immunotherapy, possibly due to its immunosuppressive phenotype.

References

1. Babjuk M, Burger M, Zigeuner R, Shariat SF, van Rhijn BW, Comperat E, et al. EAU Guidelines on Non-Muscle-invasive Urothelial Carcinoma of the Bladder: Update 2013. Eur Urol 2013.

2. Ehdaie B, Sylvester R and Herr HW. Maintenance Bacillus Calmette-Guerin Treatment of Non-muscle-invasive Bladder Cancer: A Critical Evaluation of the Evidence. Eur Urol 2013.

3. BÖHLE A and BRANDAU S. Immune Mechanisms in Bacillus Calmette-Guerin Immunotherapy for Superficial Bladder Cancer. Journal of Urology 2003; 170: 964-969.

4. Alexandroff AB, Nicholson S, Patel PM and Jackson AM. Recent advances in bacillus Calmette-Guerin immunotherapy in bladder cancer. Immunotherapy 2010; 2(4): 551-60.

5. Qian BZ and Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell 2010; 141(1): 39-51.

6. Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, et al. Macrophage polarization in tumour progression. Semin Cancer Biol 2008; 18(5): 349-55.

7. Mantovani A, Sozzani S, Locati M, Allavena P and Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002; 23(11): 549-55.

8. Mantovani A and Locati M. Tumor-associated macrophages as a paradigm of macrophage plasticity, diversity, and polarization: lessons and open questions. Arterioscler Thromb Vasc Biol 2013; 33(7): 1478-83.

9. Shimura S, Yang G, Ebara S, Wheeler TM, Frolov A and Thompson TC. Reduced infiltration of tumor-associated macrophages in human prostate cancer: association with cancer progression. Cancer Res 2000; 60(20): 5857-61.

10. Ajili F, Kourda N, Darouiche A, Chebil M and Boubaker S. Prognostic value of tumor-associated macrophages count in human non-muscle-invasive bladder cancer treated by BCG immunotherapy. Ultrastruct Pathol 2013; 37(1): 56-61.

11. Takayama H, Nishimura K, Tsujimura A, Nakai Y, Nakayama M, Aozasa K, et al. Increased infiltration of tumor associated macrophages is associated with poor prognosis of bladder carcinoma in situ after intravesical bacillus Calmette-Guerin instillation. J Urol 2009; 181(4): 1894-900.

12. Ayari C, LaRue H, Hovington H, Decobert M, Harel F, Bergeron A, et al. Bladder tumor infiltrating mature dendritic cells and macrophages as predictors of response to bacillus Calmette-Guerin immunotherapy. Eur Urol 2009; 55(6): 1386-95.

13. Tang X. Tumor-associated macrophages as potential diagnostic and prognostic biomarkers in breast cancer. Cancer Lett 2013; 332(1): 3-10.

14. Shabo I and Svanvik J. Expression of macrophage antigens by tumor cells. Adv Exp Med Biol 2011; 714: 141-50.

15. Askeland EJ, Newton MR, O'Donnell MA and Luo Y. Bladder Cancer Immunotherapy: BCG and Beyond. Adv Urol 2012; 2012: 181987.

16. Luo Y. Blocking IL-10 enhances bacillus Calmette-Guerin induced T helper Type 1 immune responses and anti-bladder cancer immunity. Oncoimmunology 2012; 1(7): 1183-1185.

17. *Solinas G*, G*ermano G*, *Mantovani A* and *Allavena P*. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *Journal of Leukocyte Biology* 2009; 86: 1065-1073.

18. Luo Y, Chen X and O'Donnell MA. Role of Th1 and Th2 cytokines in BCG-induced IFN-gamma production: cytokine promotion and simulation of BCG effect. Cytokine 2003; 21(1): 17-26.

19. Murdoch C and Lewis CE. Macrophage migration and gene expression in response to tumor hypoxia. Int J Cancer 2005; 117(5): 701-8.

20. Oosterlinck W, van der Meijden A, Sylvester R, Böhle A, Rintala E, Solsona Narvón E, et al., *Guidelines on TaT1 (Non-muscle invasive) Bladder Cancer*, in *EAU Guidelines 2006*, E.G. Office, Editor. 2006, EAU Guidelines Office: Arnhem, The Netherlands.

21. Zuiverloon TCM, Nieuweboer AJM, Ve´kony H, Kirkels WJ, Bangma CH and Zwarthoff EC. Markers Predicting Response to Bacillus Calmette-Gue´rin Immunotherapy in High-Risk Bladder Cancer Patients: A Systematic Review. European Urology 2011: 1-18.

22. Lima L, Dinis-Ribeiro M, Longatto-Filho A and Santos L. Predictive biomarkers of bacillus calmette-guerin immunotherapy response in bladder cancer: where are we now? Adv Urol 2012; 2012: 232609.

23. Medrek C, Pontén F, Jirström K and Leandersson K. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. BMC Cancer 2012; 12: 306.

24. Suyani E, Sucak GT, Akyurek N, Sahin S, Baysal NA, Yagci M, et al. Tumor-associated macrophages as a prognostic parameter in multiple myeloma. Ann Hematol 2013; 92(5): 669-77.

25. Komohara Y, Niino D, Saito Y, Ohnishi K, Horlad H, Ohshima K, et al. Clinical significance of CD163 tumor-associated macrophages in patients with adult T-cell leukemia/lymphoma. Cancer Sci 2013.

26. Dannenmann SR, Thielicke J, Stockli M, Matter C, von Boehmer L, Cecconi V, et al. Tumor-associated macrophages subvert T-cell function and correlate with reduced survival in clear cell renal cell carcinoma. Oncoimmunology 2013; 2(3): e23562.

27. Hanada T, Nakagawa M, Emoto A, Nomura T, Nasu N and Nomura Y. Prognostic value of tumor-associated macrophage count in human bladder cancer. Int J Urol 2000; 7(7): 263-9.

28. Zhang QW, Liu L, Gong CY, Shi HS, Zeng YH, Wang XZ, et al. Prognostic significance of tumor-associated macrophages in solid tumor: a meta-analysis of the literature. PLoS One 2012; 7(12): e50946.

29. Chen WT, Hung WC, Kang WY, Huang YC, Su YC, Yang CH, et al. Overexpression of cyclooxygenase-2 in urothelial carcinoma in conjunction with tumor-associated-macrophage infiltration, hypoxia-inducible factor-1alpha expression, and tumor angiogenesis. APMIS 2009; 117(3): 176-84.

30. Chai CY, Chen WT, Hung WC, Kang WY, Huang YC, Su YC, et al. Hypoxia-inducible factor-1alpha expression correlates with focal macrophage infiltration, angiogenesis and unfavourable prognosis in urothelial carcinoma. J Clin Pathol 2008; 61(5): 658-64.

CHAPTER III

Conclusion and Future Perspectives

Conclusion and Future Perspectives

From this study we can conclude that subtype M2 macrophages ($CD163^{+}$) is a better indicator of treatment failure than the counts of overall macrophage and indicates that M2 macrophages under normoxic conditions may exert an inhibitory effect on BCG immunotherapy, possibly due to its immunosuppressive phenotype.

There remain no doubts that macrophages have a major importance in tumor development. Depending on the mode of activation, macrophages can promote or suppress tumors. The tumor microenvironment is one of the most important factors to take into account in the interaction of macrophages with tumor cells.

It is important to reproduce the same study with a larger population as well as making the macrophages counts reproducible for example by using an automated count software. In the further studies will be important to assess in more detail the importance of hypoxia in the modulation of macrophage or other microenvironmental factors. Evaluate what exact cytokines (IL-10, VEGF) from tumor intervening in the role of TAMs, what way can they trigger your response, its location in the tumor and whether can be targeted for therapeutic strategies.